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Update section

Short communication

Supported PCR: an efficient procedure to amplify sequences flanking a known DNA segment

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Abstract

We describe a novel modification of the polymerase chain reaction for efficient *in vitro* amplification of genomic DNA sequences flanking short stretches of known sequence. The technique utilizes a target enrichment step, based on the selective isolation of biotinylated fragments from the bulk of genomic DNA on streptavidin-containing support. Subsequently, following ligation with a second universal linker primer, the selected fragments can be amplified to amounts suitable for further molecular studies. The procedure has been applied to recover T-DNA flanking sequences in transgenic tomato plants which could subsequently be used to assign the positions of T-DNA to the molecular map of tomato. The method called supported PCR (sPCR) is a simple and efficient alternative to techniques used in the isolation of specific sequences flanking a known DNA segment.

Plant DNA segments flanking T-DNA or transposon inserts can be obtained via conventional library construction, by means of plasmid rescue or by PCR methods. Production and screening of DNA libraries are quite laborious and time-consuming especially when large plant populations are involved in a T-DNA or transposon tagging experiment. Plasmid rescue although being effective is limited by the necessity within the tag of a plasmid origin of replication and a marker gene selective in *Escherichia coli* which is not always the case. Conceptionally, PCR serves as a most promising and powerful tool to rapidly recover flanking DNA sequences [1–2].

PCR amplification of flanking DNA fragments is, however, problematic because of the lack of sequence information for one side of the fragment to be amplified. Only one of the primers to be used in the amplification process can be designed, while the second primer should be constructed upon some manipulations of the target DNA. Different approaches have been developed to solve this problem. Nevertheless, in our hands the commonly used inverse PCR procedure (iPCR)

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[3] often gives dissatisfaction with results. Self-circularization of multiple DNA fragments is problematic and might generate concatemers or involve any other DNA fragment ligated to the target template fragment. New PCR modifications, such as single-specific-primer PCR [4, 5], oligo-cassette-mediated PCR [6, 7] or targeted gene walking PCR [8], have helped to overcome some of the problems of iPCR, mainly by avoiding self-circularization of template DNA. Still these modifications of PCR either are not reliable in studies of complex eukaryotic genomes [4, 5, 7] or cannot be applied so easily [6–8].

We set out to develop an alternative procedure for which limited sequence information is the only prerequisite to produce micrograms of unknown sequence that occurs either upstream or downstream from the original position.

The principle of the supported PCR procedure is outlined in Fig. 1. Plant DNA fragments are obtained by separate digestion with different restriction endonucleases without a priori knowledge of the sequence beyond the original target site. Primers designed on the basis of known sequence information serve to biotinylate the desired fragments selectively during the *Taq* polymerase mediated extension of the second strand to the nearest cleaved site. In the next step biotinylated fragments are immobilized on streptavidin-agarose and the bulk of genomic DNA is removed as a result of subsequent washings. The selectively enriched population of target fragments contains one blunt end. We have used the same approach as described for ligation mediated PCR [9] to equip this end in a defined orientation with a double-stranded linker primer. Exponential PCR amplification is then possible to multiply the fragments of interest.

The procedure itself includes the following steps.

Step 1. Incorporational biotinylation of a flanking fragment of interest, directed by a primer complementary to a known DNA segment. 2–4 μg of digested plant DNA was subjected to biotinylation in a 50 μl reaction mixture under the following conditions: 1 μM of target-specific primer (P1), 0.5 unit of *Thermus aquaticus Taq* DNA polymerase (Boehringer Mannheim Biochemica), 200 μM of dATP, dCTP and dGTP, 150 μM of dTTP and 50 μM of bio-11-dUTP (Sigma) in the buffer recommended by the supplier. Polymerization was achieved after denaturation of template DNA for 5–6 min at 94 °C, followed by annealing with primer P1 during 2 min at 55 °C and extension for 10 min at 72 °C with subsequent cooling to 4 °C. The reaction was stopped by addition of EDTA, pH 8.0, to a final concentration of 50 mM.

Step 2. Selective isolation of biotinylated DNA molecules by immobilization on streptavidin-containing support. Immobilization of biotinylated DNA molecules was achieved directly by the addition of approximately 12–14 μl of streptavidin-agarose (Sigma) to the reaction mixture (step 1), mixing and incubation for 30 min at 37 °C. Agarose particles containing DNA fragments were then washed 3 times with TBS buffer, containing 25 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.1 mM EDTA, and 3 times with water.

Step 3. Attachment of universal linker primer to the immobilized enriched population of DNA molecules. Ligation of template DNA with double-stranded linker primer (LP) was performed in a total volume of 25 μl, containing approximately 600 pM of linker primer, 8 units of T4 DNA ligase (Boehringer Mannheim Biochemica) in the ligation buffer recommended by the manufacturer for 14–16 h at 12–14 °C.

Step 4. Exponential amplification of the fragment of interest now being between two primers, the universal linker primer and the target specific primer. Agarose particles were washed once with TBS buffer and 2 times with water and subjected to PCR amplification in the presence of the long oligomer of the linker primer (LP1) and the target-specific primer (P1). PCR reactions were performed under standard conditions (50 μl) using 1–1.5 units of *Taq* DNA-polymerase, 1 μM of each primer and 200 μM of dNTPs in the polymerase buffer recommended by the manufacturer.

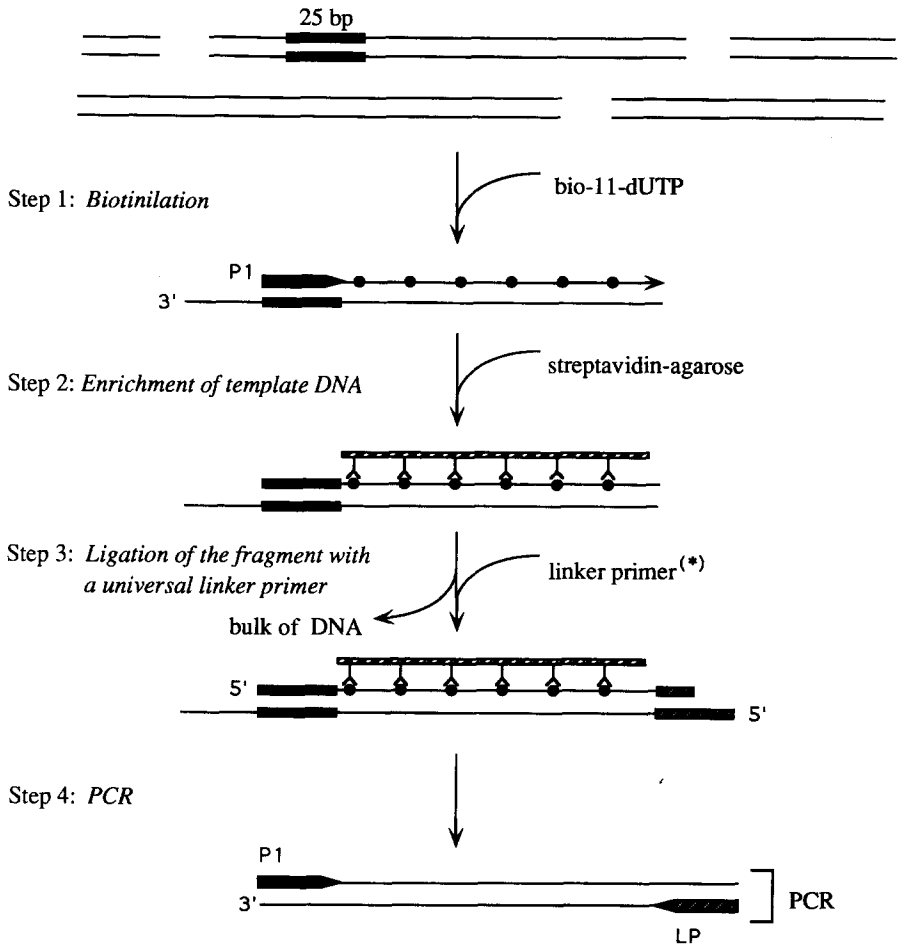

Fig. 1. Generalized schematic representation of the supported PCR technique. The sPCR procedure consists of four steps being performed in one and the same reaction vessel. These steps include biotinylation of fragments of interest in complex DNA mixture (step 1), selective enrichment of the template DNA on streptavidin-agarose (step 2), equipment of the fragment by the second primer (step 3) and amplification (step 4). A detailed explanation and discussion of the different steps can be found in the text. The universal double-stranded linker primer (LP) indicated by (*) at step 3 consisted of two oligomers LP1 (25 bp) and LPs (11 bp). The short oligomer was phosphorylated by T4 polynucleotide kinase. Annealing of an equimolar mixture of these oligomers (94 °C to 4 °C) resulted in primers with one blunt end and a 14 bp single-stranded 5′ overlap. Oligomer sequences are: LP1: 5′-OH-ACCCG TGGAT CAGTA CCGCG ACTTG-OH-3′, LPs: 5′-P-CAAGT CGCGG T-OH-3′.

Amplifications were achieved after denaturation of template DNA at 94 °C for 4 min and 35 repeated cycles of 30 s denaturation at 94 °C, 1 min annealing at 55 °C and 1.5–3 min at 72 °C followed by a final 5 min extension step and cooling to 4 °C using the Perkin Elmer Cetus Thermal Cycler. The reaction products were directly analysed on 2% SeaKem agarose gels. In order to get sufficient amplified product for cloning/sequencing or direct use as a hybridization probe without intervening cloning steps, an aliquote of the first amplification round can be used for reamplification using a 'nested' target specific primer (P2, see Fig. 2, panel A) and the long oligomer of the linker primer (LP1).

Four transgenic tomato plants containing either single (AAT6514-30/-33/-02) or three different (AAT6514-44) T-DNA insertions (according to Southern blot analysis, data not shown) were used as a model system to apply the sPCR strat-

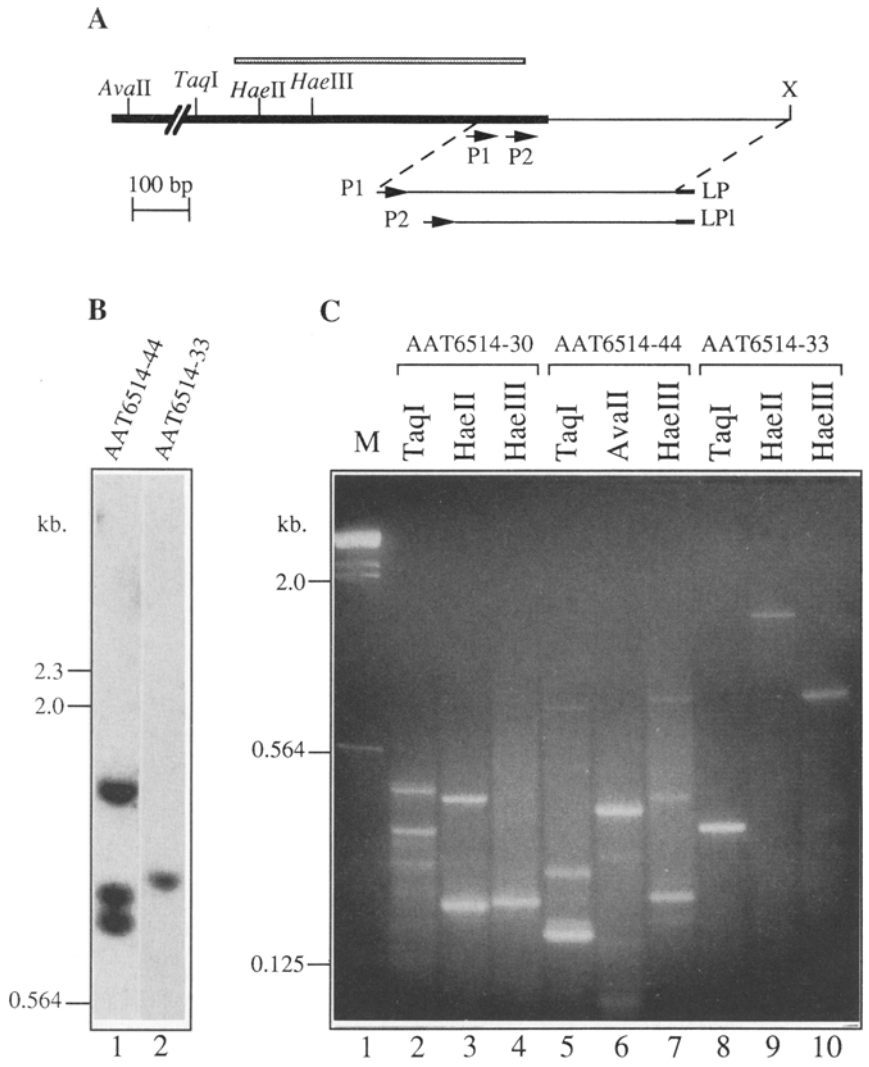

Fig. 2. Supported PCR applied to recover plant DNA sequences flanking the boundary of integrated T-DNA. Panel A: schematic representation of a T-DNA (thick line)/plant DNA (thin line) junction and strategy to generate template DNA for sPCR. For simplicity only one DNA strand is shown. Sites for restriction endonucleases used to obtain fragments of interest are indicated within the T-DNA, whereas X indicates the corresponding unknown positions within plant DNA. Note, that the restriction site for *Ava* II in the T-DNA is 1.03 kb from the boundary. Target-specific primers used for biotinylation (P1) and amplification (P1 and P2) are based on T-DNA right border sequence information [11], are: Pl: 5′-CGGCT TGTCC CGCGT CATCG GC-3′, P2: 5′-CCCCT AGGGA TTGTC GTTTC CCGCC TTCAG-3′. P1 is located 82 bp and P2-18 bp upstream from the boundary. LP1-long oligomer of the universal linker primer. The dashed line represents the hybridization probe used in Southern blot analysis (panel B). Panel B: Southern blot analysis of plant DNA digested by *Taq* I and subjected to sPCR further on (see panel C). Lane 1-plant AAT6514-44 (three T-DNA inserts), lane 2-AAT6514-33 (single T-DNA) DNA digests separated on 1% agarose gels and hybridized with a randomly primed (BRL) probe specific to the T-DNA border. Observed sizes for T-DNA/plant DNA junctions were: 1.35 kbp, 850, 750 (lane 1) and 890 (lane 2) bp. Since the *Taq* I cleavage site is 626 bp apart from the boundaries, corresponding sizes of the DNA templates to be amplified by sPCR in these cases are estimated to be about 780, 260, 170 (AAT6514-44) and 310 (AAT6514-33) bp, respectively. Panel C: sPCR products generated for transgenic tomato plants. DNA isolated from three transformants was digested with different restriction endonucleases as indicated and subjected to sPCR amplification. T-DNA flanking regions were selectively biotinylated with the help of the P1 primer. This primer and the long oligomer of the linker primer (LP1) were used during the first round of amplification. About 1/5000 dilution of the obtained products was used for the second round of amplification (P2 and LP1 primers). Half a volume of the final products (25 μl) were analysed on a 2% agarose gel. λ DNA digested by *Hin*d III served as molecular weight marker (lane 1).

egy. A schematic representation of the T-DNA boundary region together with an illustration of the way in which sPCR templates were generated is shown in Fig. 2, panel A. The selection of restriction enzymes to prepare DNA templates sized to be optimal for efficient amplification was done on the basis of Southern blot analysis with a T-DNA right-border probe. As demonstrated by the example in Fig. 2, panel B, *Taq* I can be suitable to generate three different DNA templates for plant AAT6514-44 and one for AAT6514-33. Figure 2, panel C, summarizes the sPCR products obtained for three of these transgenic plants. Four base pair cutting enzymes showed to be more preferable for generation of templates reflecting the T–DNA copy number, especially when several flanking DNA fragments have to be amplified at once (compare lanes 5, 8 (*Taq* I), lanes 4, 7, 10 (*Hae* III) and lane 6 (*Ava* II) in Fig. 2, panel C).

The number of major sPCR products generated for plant AAT6514-30 (single T-DNA insertion) was more than expected (Fig. 2, panel C, lanes 2, 3). Based on contiguity of the plant DNA and T-DNA we presumed that the upper *Taq* I and *Hae* II fragments (416 and 387 bp, respectively) might represent more distal cleavage sites. Sequence analysis of the upper *Taq* I (416 bp) fragment (Fig. 2, panel C, lane 2) revealed the occurrence of an additional internal *Taq* I site as well as *Hae* II and *Hae* III sites in the plant DNA. This implies either incomplete DNA digestion during preparation of template DNA or methylation of certain restriction sites. Other sPCR products, such as the 324 bp (lane 1), 266 and 165 bp (lane 5) and 306 bp (lane 7) fragments were gel purified, subsequently cloned into the vector pTZ19R and sequenced by Sequenase version 2.0 (United States Biochemical) using universal M13 (−21) direct and reverse primers (to be published elsewhere). In all these and other cases (plant AAT6514-02) sequence analysis revealed the authenticity of sPCR products and flanking nature of amplified plant DNA. As an additional control, plant DNA blots, used for hybridization analysis with the T-DNA right border probe (as in Fig. 2, panel B) were reprobed with some of the sPCR products. The obtained banding pattern confirmed the identity of the plant DNA/T-DNA junctions (results not shown).

Although the supported PCR method as presented yields specific products as major fragments upon amplification with the T-DNA specific primers, there are some minor fragments visible in Fig. 2, panel C. The partially phosphorylated linker primer used during the ligation step (see legend to Fig. 2, panel 1) is able to generate concatemer fragments upon amplification as confirmed by sequence analysis of some of these products (results not shown). Nevertheless, these products do not interfere with specific ones and their appearance can be avoided by more careful washings of agarose particles after the ligation step or by the use of unphosphorylated linker primer, although the efficiency of ligation in the latter case is lower.

The isolation of plant DNA segments flanking T-DNA as it was presented here allowed us to locate the corresponding T-DNA positions on the RFLP map of tomato. The RFLP mapping was done essentially as described [10], except that sPCR products were directly used as hybridization probes. T-DNAs from plants AAT6514-33 and -02 were located on chromosome I, pos. 4 and chromosome 6, pos. 42, respectively. One T-DNA from plant AAT6514-44 was assigned to chromosome 1, pos. 136, whereas a second T-DNA was in repetitive plant DNA and could not be mapped. No RFLP could be detected by plant DNA fragments flanking the third T-DNA insert in plant AAT6514-44 and the T-DNA in plant AAT6514-30.

By using the sPCR procedure we were able to amplify T-DNA flanking plant genomic single-copy sequences up to at least 1.6 kb (Fig. 2, panel B). Other potential applications of sPCR include direct gene isolation on the basis of very limited sequence information, chromosomal walkings without intervening steps like cloning and screening and diagnostic applications like detection of DNA polymorphisms.

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